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# Arsonium-containing Lipophosphoramides, Poly-functional Nano-carriers for Simultaneous Antibacterial Action and Eukaryotic Cell Transfection

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**Summary**



**Abbreviations**: AA, antibacterial activity; BGTC, bis(guanidinium)-tren cholesterol; BSV, Brest synthetic vector; CF, cystic fibrosis; CFU, colony forming unit; CR, charge ratio; Ec, *Escherichia coli*; i.p., intraperitoneal; i.v., intravenous; MIC, minimal inhibitory concentration; MBC, minimal bactericidal concentration; LB, Luria Broth; LFM, lipofectamine; LX, lipoplex; MRSA, methicillin-resistant *Staphylococcus aureus*; Pa, *Pseudomonas aeruginosa*; pDNA, plasmid DNA; PEI, poly(ethylenimine); RPM, rotation per minute; RLU, relative light unit; RT, room temperature; Sa, *Staphylococcus aureus*; TE, transfection efficiency.

**SI1. Detailed structure of the cationic lipids**

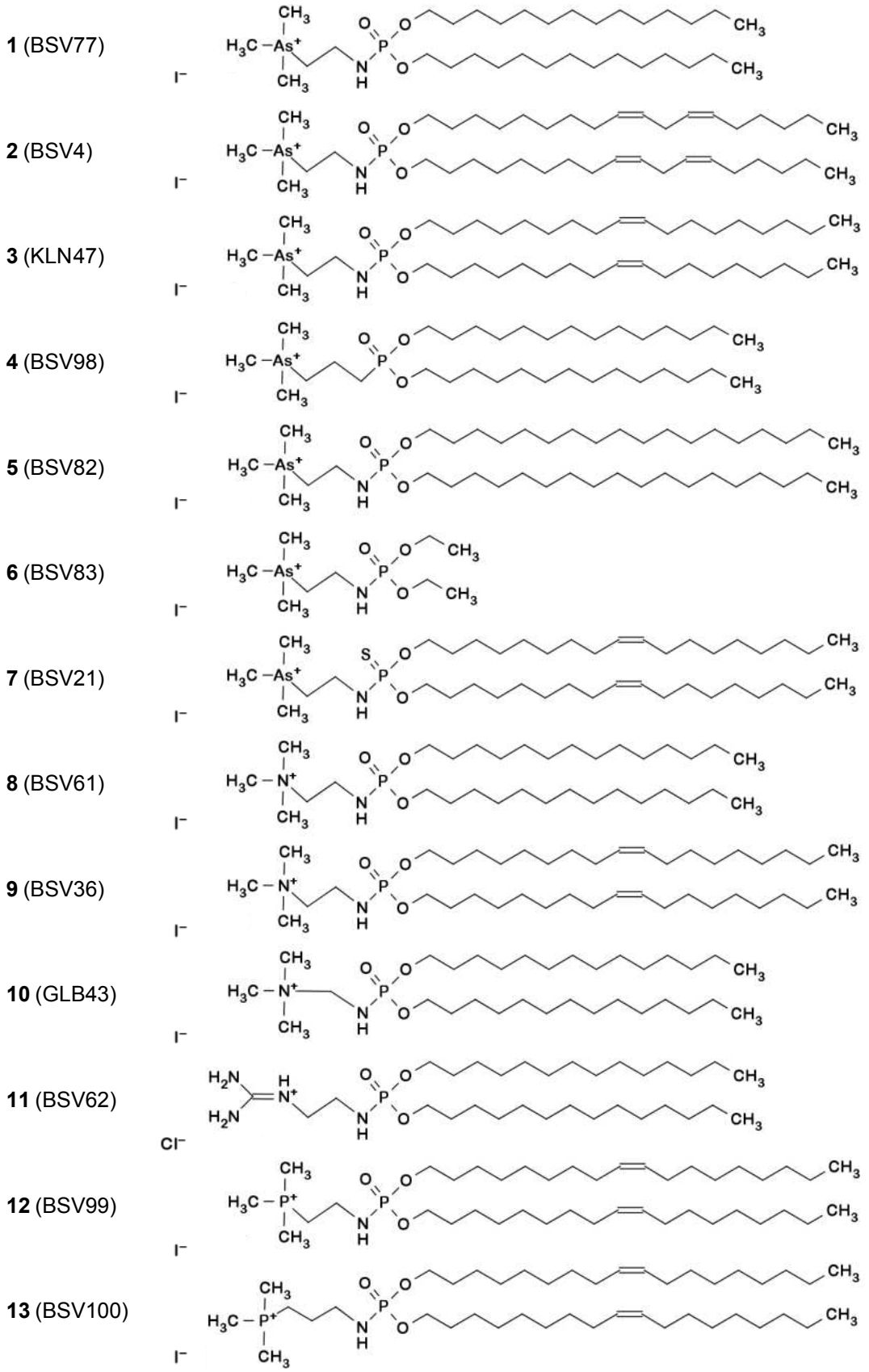


**Figure S1.** Chemical structure of the various representative cationic lipids evaluated herein.

**SI2. X-ray crystal structure of compound 6**

Single crystal Diffraction data were collected at 170 K on an Xcalibur 2 diffractometer (Oxford Diffraction) using graphite-monochromated Mo Kα radiation (λ = 0.71073 Å). The structure was solved by direct methods and successive Fourier difference syntheses and was refined on $F^2$ by weighted anisotropic full-matrix least-squares methods.[1] All the non-hydrogen atoms were refined anisotropically. All the hydrogen atoms were calculated for both structures and therefore included as isotropic fixed contributors to Fc. The thermal ellipsoid drawings were made with the ORTEP program.[2] Data collection and data reduction were done with the CRYSALIS-CCD and CRYSALIS-RED programs.[3] All other calculations were performed using standard procedures (embedded with WinGX suite of programs).[4]

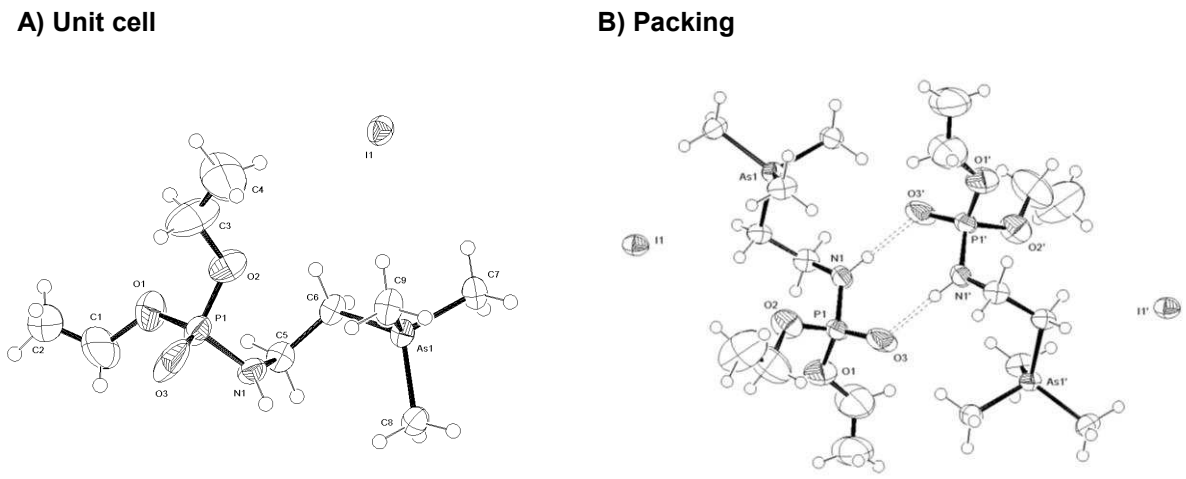


**Figure S2.** X-Ray crystal-structure (ORTEP plot – Ellipsoids are represented at the 50% probability level) of 2-(*O,O*-diethoxyphosphoramidyl)-ethyltrimethylarsonium iodide (compound **6**) (Data collected at 170 °K, space group $P2_1/n$ (14); selected data d(Å): P1-O3 1.450(5); P1-O2 1.556(5); P1-O1 1.552 (5); P1-N1 1.611 (5); As1-C6 1.931(5); As1-C8 1.898(5)). A) Asymmetric unit of **6**; B) Two units of **6** arranged as a dimer stabilized by hydrogen bonds (d(Å) N1-H1 0.88; H1…O3 1.96; N1…O3 2.835(6)).

## SI3. Physicochemical characteristics of liposomal solutions

**Table S1.** Size and zeta potential of liposomal solutions[a)] prepared with the various representative cationic lipids evaluated in this study.

| Compounds | Mean Particle Sizes (nm) | Poly Index[b)] | Zeta Potential (mV) |
|---|---|---|---|
| **1** (BSV77) | 233 | 0.22 | + 38 |
| **2** (BSV4) | 224 | 0.21 | + 41 |
| **3** (KLN47) | 242 | 0.36 | + 49 |
| **4** (BSV98) | 142 | nd | + 35 |
| **5** (BSV82) | 190 | 0.49 | + 53 |
| **7** (BSV21) | 241 | 0.15 | + 60 |
| **8** (BSV61) | 179 | 0.19 | + 40 |
| **9** (BSV36) | 267 | 0.36 | + 33 |
| **10** (GLB43) | 205 | 0.41 | + 33 |
| **11** (BSV62) | 145 | 0.29 | + 40 |
| **12** (BSV99) | nd | nd | nd |
| **13** (BSV100) | nd | nd | nd |

[a)] Liposomal solutions were prepared at 1.5 mM. Of note, at this concentration, compound **6** does not form any aggregates in water; [b)] Poly Index, poly-dispersity index; nd, not determined.

## SI4. Antibiotic resistance profile of the bacterial strains

**Table S2.** Antibiotic resistance profile[a)] of the bacterial strains.

| Gram positive strains[b)] | Antibiotic[c)] | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Oxa | Amo | Clav | Gent | Net | Tob | Ami | Kan | Dox | Ery | Lin | Pri | Pef | Cot | Fus | Tei | Van | Lin | Mup |
| Sa RN4220 | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| Sa Newman | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S | S |
| Sa N315 | **R** | **R** | **R** | S | S | **R** | **R** | **R** | S | **R** | **R** | S | S | S | S | S | S | S | S |

Number of antibiotic resistances per strain: Sa RN4220, 0 out of 19; Sa Newman, 0 out of 19; Sa N315, 8 out of 19.

| Gram negative strains[b)] | Antibiotic[c)] | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Tic | Clav | Pip | Taz | Aza | Ceft | Cefs | Cefo | Imi | Gen | Tob | Net | Ami | Lev | Cif | Fos | Cot | Col | Tem |
| Ec MG1655 | **R** | **R** | **R** | S | S | S | **R** | S | S | S | S | S | S | S | S | S | nd | S | nd |
| Pa 130709 | S | S | S | S | S | S | **R** | S | **R** | L | S | **R** | L | nd | S | **R** | **R** | **R** | **R** |
| Pa 240709 | **R** | **R** | **R** | **R** | **R** | **R** | **R** | **R** | S | **R** | **R** | **R** | **R** | nd | S | **R** | **R** | S | nd |

Number of antibiotic resistances per strain: Ec MG1655, 4 out of 17; Pa 130709, 7 out of 18; Pa 240709, 14 out of 17.

[a)] as determined with the antibiogram reference method; [b)] Sa, *S. aureus*; Ec, *E. coli*; Pa, *P. aeruginosa*; [c)] Antibiotic abbreviations: Ami, amikacin; Amo, amoxicilline; Aza, aztreonam; Cefo, cefoperazon; Cefs, cefsulodin; Ceft, ceftazidim; Cif, ciprofloxacin; Clav, amoxicilline+clavulinic acid; Col, colistin; Cot, cotrimoxazole; Dox, doxicillin; Ery, erythromycin; Fos, fosfomycin; Fus, fusidic acid; Gent, gentamicin; Imi, imipenem; Kan, kanamycin; Lev, levofloxacin; Lin, lincomycin; Lin, lincomycin; Mup, mupirocin.; Net, netilmicin; Pef, pefloxacin; Pip, piperacillin; Pri, pristinamycin; Taz, piperacillin+tazobactam; Tei, teicoplanine; Tem, temocillin.; Tob, tobramycin; Van, vancomycin; Oxa, oxacilline; Tic, ticarcilline. R, resistant; L, limit; S, sensitive; nd, not determined.

**SI5. Drug resistance study**

In order to investigate whether or not arsonium-containing cationic lipids might select for drug-resistant isolates, the MRSA strain N315 was cultivated for serial passages on half-MIC of **2**, MIC values being re-evaluated every 24 h. As a positive control, the antibiotic norfloxacin (NFX) was evaluated in parallel in the same way.

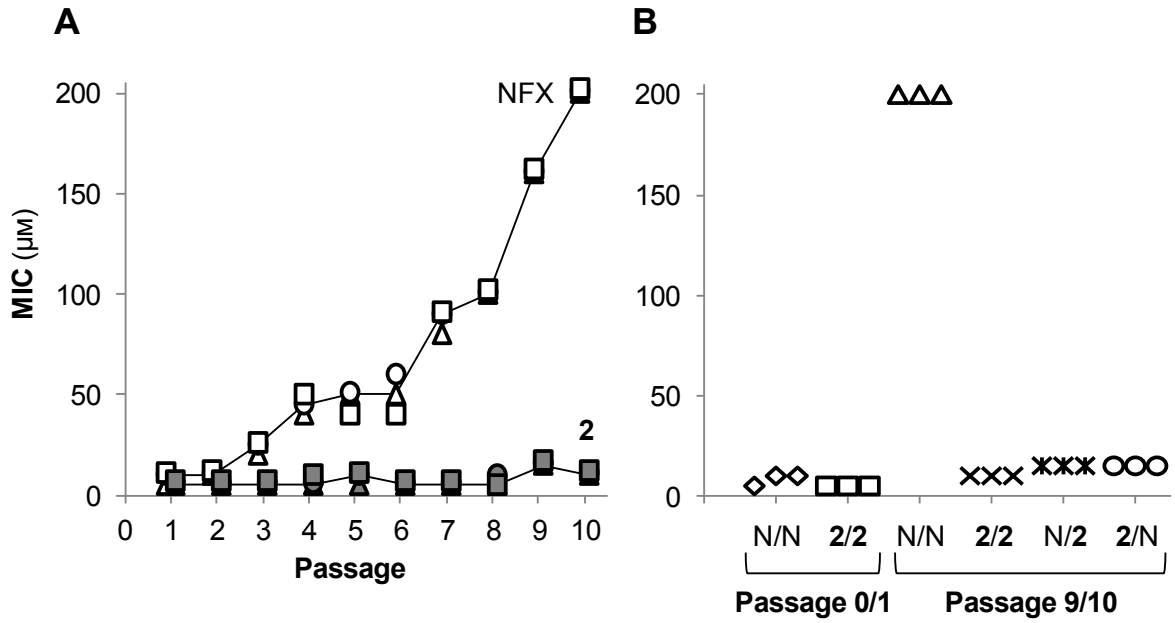


**Figure S3.** Evaluation of the ability of a methicillin resistant *S. aureus* strain to develop resistances towards an antibiotic or an arsonium-containing cationic lipid. (**A**) Minimal inhibitory concentrations (MIC) were determined while cultivating the strain N315 for 10 passages (one each 24 h) in presence of either Norfloxacin (NFX, N) or **2**. Bacteria growing at one-half of the MIC were used to prepare bacterial dilution ($10^6$ to $10^7$ CFU/mL) used for subsequent passage. (**B**) As controls, at passage 9 to 10, bacteria previously cultivated in presence of one drug were exposed to the other one (n=3).

While the susceptibility of bacteria towards **2** did not change after 10 passages, a strong increase in MIC of NFX was already detected after 3 passages, with a more than 20-fold increase in MIC measured after 10 days. This supports that arsonium-containing cationic lipids should not participate in the development of drug-resistant strains.

**SI6. Stability of a naked DNA under sterile or infected conditions**

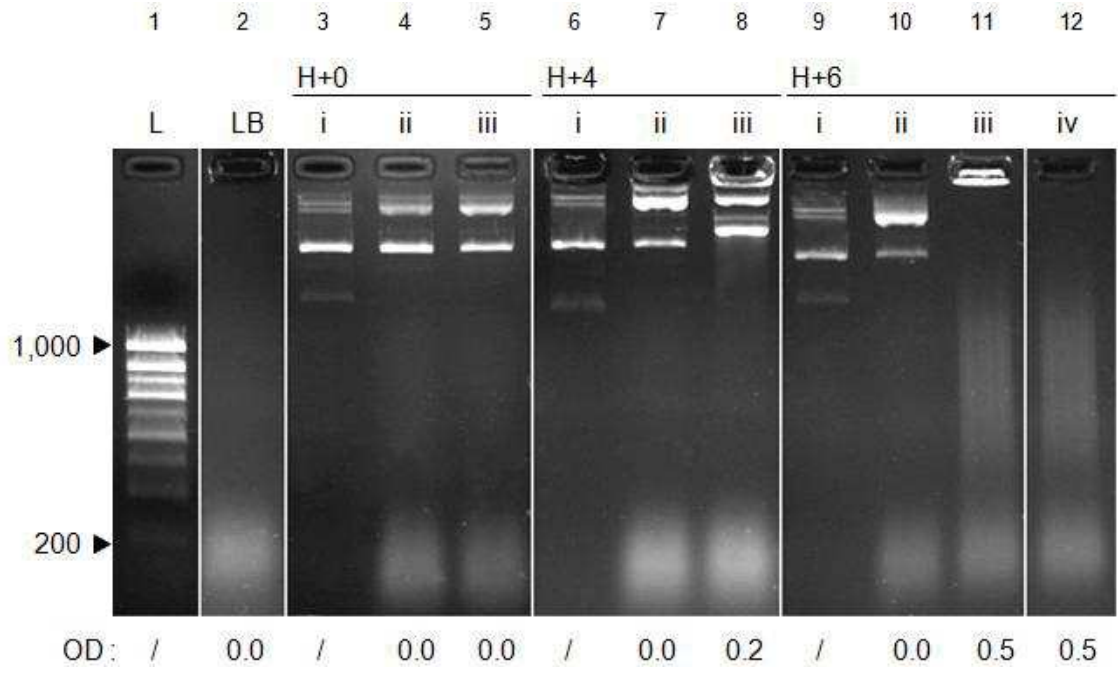


**Figure S4.** Stability of a naked pDNA when diluted in a culture medium either sterile or inoculated with a bacterial strain. A pDNA was diluted either in water (i), in sterile LB broth (ii) or in LB broth inoculated with the *S. aureus* strain RN4220 (iii) and then incubated at 37 °C for 6 h. At 3 time points (H), optical densities at 650 nm were measured (for assessing bacterial growth) and an aliquot of each condition was analysed by agarose gel electrophoresis (for assessing DNA integrity). L, 100 bp DNA ladder (Smartladder SF, Eurogentec); LB, LB broth alone; i, pDNA in water; ii, pDNA in sterile LB broth; iii, pDNA in inoculated LB broth.

- Lane 2: The lower diffused band visible at the bottom of wells #2, #4, #5, #7, #8, #10, #11, and #12 should not be confused with DNA. It is likely that it corresponds to some LB broth components that should interact with ethidium bromide, thereby being visualised on gel.
- Lanes 3, 6 and 9: The clear delineation of distinct bands remaining unchanged at the three time points considered indicate the stability of pDNA diluted in water, at 37 °C, for at least 6 h (and up to 16 h, not shown).
- Lanes 4, 7 and 10: The dilution of pDNA in sterile LB broth leads to some conformational changes as the different forms visualised at H+0, H+4, and then H+6 are not the same. However, clear distinct bands are well observed in each case and no obvious degradation can be detected there.
- Lanes 5, 8 and 11: For conditions where DNA is diluted in an inoculated LB broth, as bacteria grow, pDNA conformational changes are first visible at H+4 then a smear with fragment sizes ranging to 200 up to >1,000 base pairs at H+6. After 16 h at 37 °C, no DNA is visualised (not shown).
- Lanes 11 and 12: Of note, the intense fluorescence observed in well #11 is related to bacteria themselves; indeed, such fluorescence was not observed in well #12 which corresponds to the electrophoresis of the supernatant collected after centrifugation (to pellet bacteria).

## SI7. Antibacterial activity and DNA compaction and protection

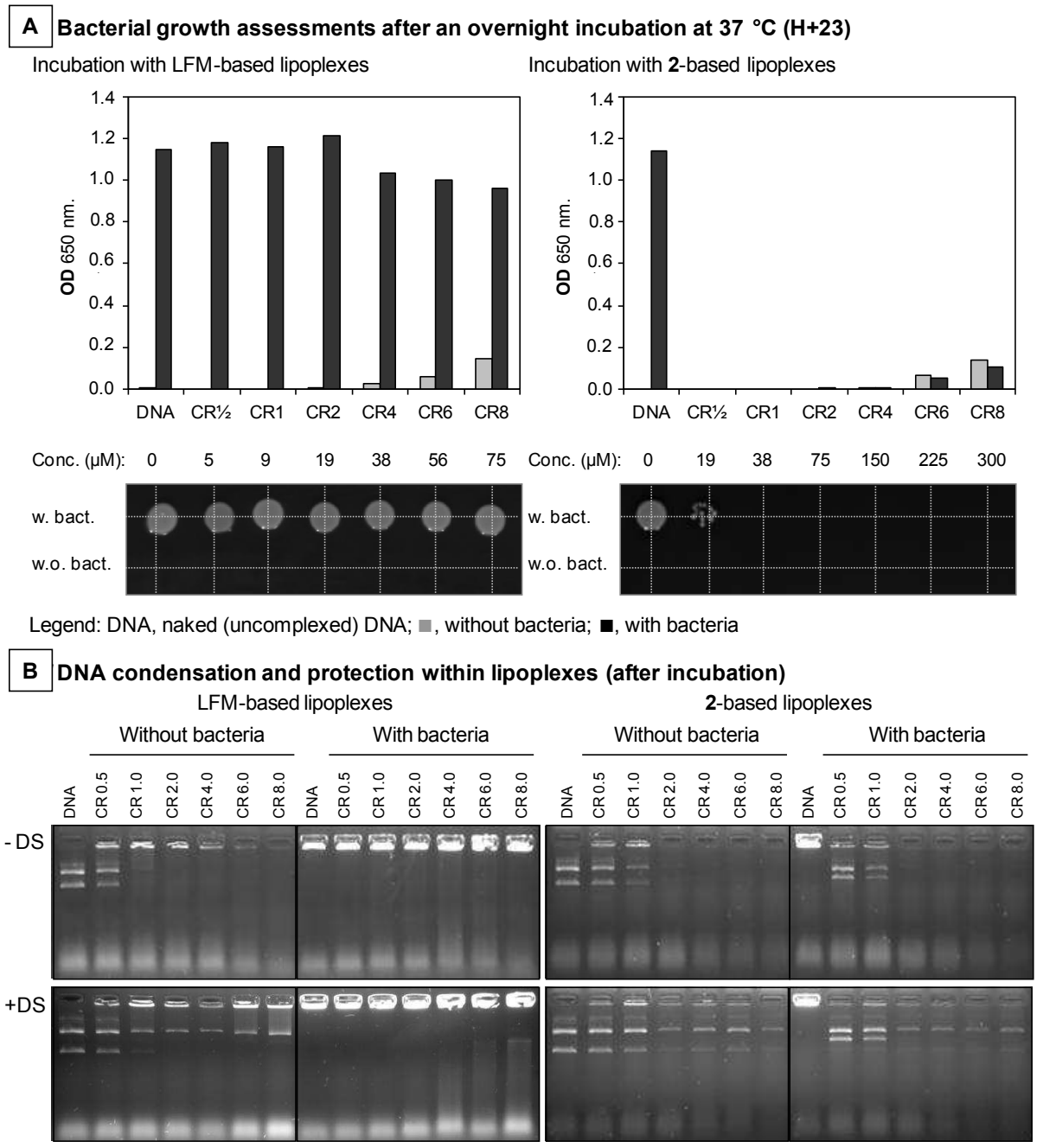


**Figure S5.** Lipoplexes prepared with an arsonium-containing lipophosphoramide can simultaneously kill bacteria and protect DNA against degradation. Lipoplexes were prepared by mixing DNA with either LFM or **2** in order to form complexes characterized with charge ratios (CR) ranging from 0.5 up to 8.0 (corresponding cationic lipid concentrations are indicated in the unit of µM). Thereafter, lipoplexes were diluted in LB broth either sterile or inoculated with the *S. aureus* strain RN4220. After an overnight incubation at 37 °C, bacterial growths were assessed spectroscopically by measuring the absorption at 650 nm and by spreading an aliquot of each condition onto nutritive non-selective agar plates (**A**); agarose gel electrophoresis were conducted to evaluate DNA condensation within lipoplexes, before and after addition of dextran sulphate (DS) (**B**). Of note, **3** provided similar results (not shown) as **2**.

## SI8. Sequential assay: antibacterial effect then *in vitro* transfection activity

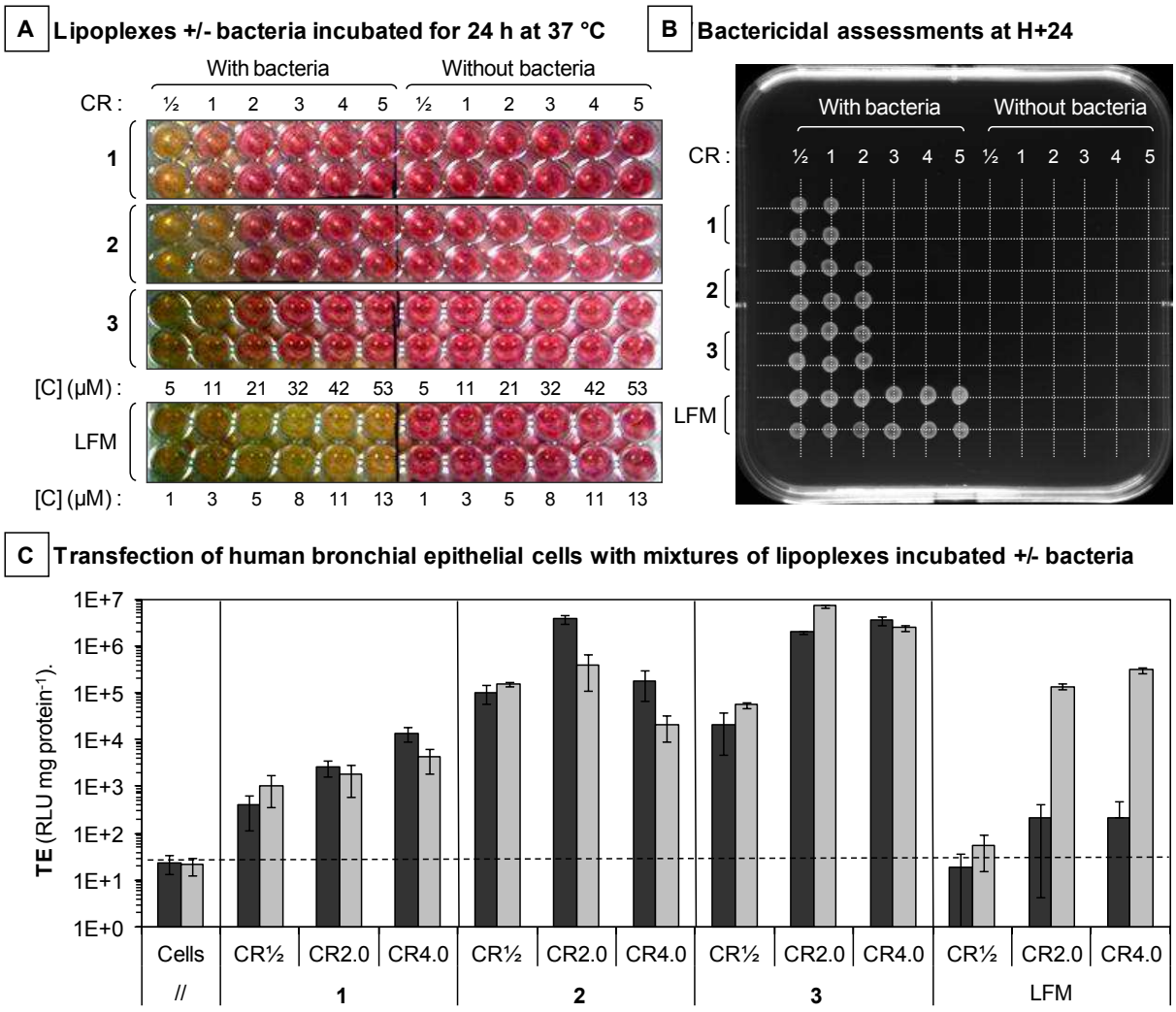


**Figure S6.** Lipoplexes prepared with an arsonium-containing lipophosphoramide can kill bacteria while remaining efficient for *in vitro* transfection of eukaryotic cells. Lipoplexes were prepared by mixing DNA with either LFM or an arsonium-containing lipophosphoramide (**1**, **2** or **3**) in order to form complexes characterized with charge ratios (CR) ranging from 0.5 up to 5.0 (corresponding cationic lipid concentrations [C] are indicated in the unit of µM). Next, lipoplexes were diluted in DMEM either sterile or inoculated with the *S. aureus* strain RN4220. After 24 h at 37 °C, a direct visual inspection of these mixtures allowed identifying conditions in which a bacterial growth had occurred (**A**). Bacterial growths were further confirmed by spreading an aliquot of each condition onto a nutritive non-selective agar plate and overnight incubation at 37 °C (**B**). Mixtures of lipoplexes with or without bacteria were then used for *in vitro* gene transfection of eukaryotic cells. The luciferase reporter system was used to evaluate the transfection efficiency measured as RLU per mg protein. The results shown were obtained by incubating the human airway epithelial cell line 16HBE with lipoplexes formed at 3 different CR. Of note, similar results were obtained when using A549 cell line (not shown) (**C**). Values are mean +/- SD with n = 3.

**SI9. Adenylate kinase measurements for monitoring bacterial growth**

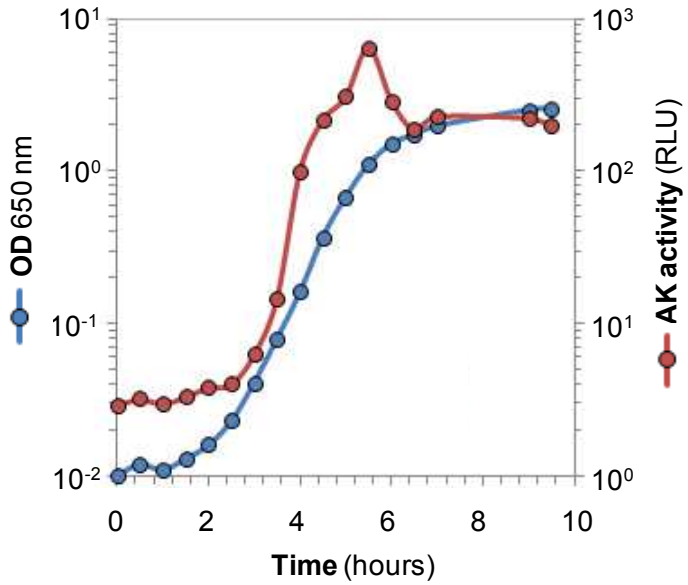


**Figure S7.** Comparative evaluation of adenylate kinase (AK) activity and medium turbidity. LB broth inoculated with the *S. aureus* strain N315 was incubated for several hours at 37 °C. At regular time interval, optical density (OD) was determined at 650 nm and a sample was collected in order to be assayed using the Toxilight kit (Lonza).

**SI10. Simultaneous assay: antibacterial effect and transfection activity in one pot**

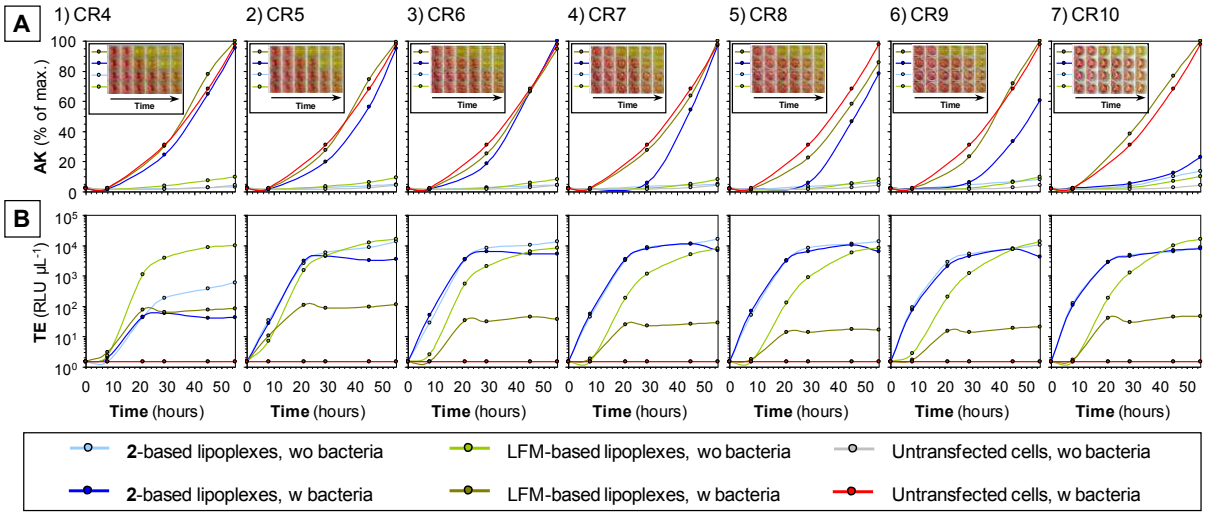


**Figure S8.** Lipoplexes prepared with an arsonium-containing lipophosphoramide can both inhibit bacteria and transfect eukaryotic cells in one pot. During 55 hours, **2**-based lipoplexes (formed at CR ranging from 4 to 10) were assayed for their simultaneous ability to (1) inhibit the growth of the bacterial strain N315 and to (2) transfect human bronchial epithelial cells both cultivated in EMEM (without any antibiotics). LFM-based lipoplexes were used as controls. Antibacterial activity was monitored by considering the adenylate kinase (AK) activity (**A**) together with variations of the medium colour (insets in **A**). Transfection efficiency (TE) was quantitated as RLU per µL of supernatant (**B**). w, with; wo, without.

## SI11. References

[1] M. Sheldrick, *SHELX97. Programs for crystal structure analysis*; Göttingen, Germany, **1997**.
[2] C. K. Johnson, *ORTEP*; Delft, The Netherlands, **1985**.
[3] *CRYSALIS PRO Software System*; Oxford Diffraction Ltd, **2007**.
[4] L. J. Farrugia, *J. Appl. Crystallogr.* **1999**, *32*, 837.